A pair of dopamine neurons mediate chronic stress signals to induce learning deficit in *Drosophila melanogaster*

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Chronic stress could induce severe cognitive impairments. Despite extensive investigations in mammalian models, the underlying mechanisms remain obscure. Here, we show that chronic stress could induce dramatic learning and memory deficits in *Drosophila melanogaster*. The chronic stress–induced learning deficit (CSLD) is long lasting and associated with other depression-like behaviors. We demonstrated that excessive dopaminergic activity provokes susceptibility to CSLD. Remarkably, a pair of PPL1-γ1pedc dopaminergic neurons that project to the mushroom body (MB) γ1pedc compartment play a key role in regulating susceptibility to CSLD so that stress-induced PPL1-γ1pedc hyperactivity facilitates the development of CSLD. Consistently, the mushroom body output neurons (MBON) of the γ1pedc compartment, MBON-γ1pedc>α/β neurons, are important for modulating susceptibility to CSLD. Imaging studies showed that dopaminergic activity is necessary to provoke the development of chronic stress–induced maladaptations in the MB network. Together, our data support that PPL1-γ1pedc mediates chronic stress signals to drive allostatic maladaptations in the MB network that lead to CSLD.

Drosophila melanogaster | chronic stress | learning and memory | dopamine neuron | depression

Stress has significant and complex effects on cognitive function. In general, these effects follow an inverted U–shaped dose–response relationship in intensity and duration. So that moderate acute stress could promote learning and memory, while chronic stress very often induces detrimental effects (1). Since chronic stress–induced learning and memory impairments are closely associated with many neural disorders, such as depression, schizophrenia, and Alzheimer's disease, understanding the underlying neurobiology is of importance for developing effective drugs and treatments (2, 3). To this end, animal models, especially mammalian models, have been extensively investigated. Current findings suggest that the effects of chronic stress on learning and memory could be influenced by many internal and external factors that involve multiple brain regions, genes, and complex mechanisms that have not yet been fully elucidated (3–6).

Stress could have consequential effects on aversive olfactory memory in *Drosophila melanogaster*. For example, moderate fast promotes long term memory (LTM) formation (7, 8), while sleep deprivation promotes forgetting and impairs memory capacity (9–11). Recent reports have shown that chronic stress can induce depression-like symptoms in *Drosophila*, as manifested by characteristic behaviors that indicate anhedonia, lack of motivation, prone to despair, and sleep disorder (12–14). However, investigation of the effect of chronic stress on *Drosophila* learning and memory is still lacking.

In the present study, we report that a 4-d chronic stress treatment (CST) effectively induces strong learning and memory deficits in *Drosophila*. We focused on the learning deficit phenotype and found that the *Drosophila* dopaminergic (DAergic) system plays an important role in modulating susceptibility to chronic stress–induced learning deficit (CSLD), suggesting that DAergic modulation is an evolutionary conserved chronic stress–coping mechanism. We pinpointed the key CSLD regulating dopamine neurons (DANs) to a pair of PPL1-γ1pedc neurons that project to the mushroom body (MB) γ1pedc compartment and further showed that MBON-γ1pedc>α/β, the output neurons of γ1pedc compartment, modulates susceptibility to CSLD as well. Imaging studies identified chronic stress–induced abnormal neural activities in learning-related neurons, which require DAergic activity during CST. Overall, our studies delineate a model that chronic stress signals can be mediated by a pair of DANs, PPL1-γ1pedc, to drive maladaptations in the MB network that lead to CSLD.

Significance

Chronic stressful life events could induce learning and memory impairments and increase the risk of developing psychiatric disorders such as depression. Understanding the underlying mechanism is critical for developing effective drugs and treatments. Here, we show that chronic stress induces learning and memory deficits in *Drosophila melanogaster*. Furthermore, the dopaminergic system is important for regulating susceptibility to chronic stress– induced learning deficit (CSLD). Significantly, a single pair of dopamine neurons, PPL1-γ1pedc neurons, are indispensable for CSLD. We show that PPL1-γ1pedc mediates chronic stress signals to induce abnormal neural activities in mushroom bodies that lead to a learning deficit. Together, these suggest that *Drosophila melanogaster* can be a powerful model organism for studying the etiology of chronic stress–induced memory impairments.

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Results

Chronic Stress Induces Olfactory Learning and Memory Deficits in *Drosophila*. To investigate the impact of chronic stress on learning and memory in adult *Drosophila*, we first established a 4-d chronic stress procedure (Fig. 1*A*). During the 4 d, groups of about 100 flies were cultured in small food vials and subjected to a 10-min mechanical shock each day. In each minute of the 10-min treatment, flies were vortexed (500 rpm) for either 5, 10, or 15 s (Video S1). The vortex was both uncontrollable and unpredictable, as it started randomly within each minute. After the 4-d CST (1 d after the last vortex), flies were tested for olfactory learning (3-min memory) or middle-term memory (3-h memory) with a classical conditioning paradigm as previously described (15, 16). The 4-d CST induced remarkable deficits in both learning (Fig. 1*B*) and middle-term memory (Fig. 1*C*), while 1-d stress treatment had no effect (*SI Appendix*, Fig. S1). The learning deficit appeared as vortex-time dependent, as 10 s of vortex shows a trend of stronger learning deficit than that of 5 s of vortex. As 15 s of vortex did not further diminish olfactory learning, we chose 10-s vortex as the standard mechanical shock condition for our CSTs. With this standard condition, we did not detect major body damage, brain cell death nor intestinal barrier damage after chronic stress (ACS) treatment (*SI Appendix*, Figs. S2–S4), indicating that the learning and memory deficits induced by chronic stress are unlikely the consequence of traumatic brain injury (13, 17–19). Importantly, the task relevant sensorimotor responses (odor acuity and shock reactivity) necessary to perform this task also appeared normal (*SI Appendix*, Fig. S5), suggesting that the standard CST specifically impaired associative learning and memory.

Chronic Stress Induces a Long-Lasting Depression-Like State in *Drosophila*. Recent reports had shown that chronic stress could induce depression-like states in *Drosophila* as evidenced by lack of motivation and anhedonia (12, 13). To determine if our CST induces a depression-like state, we tested three other behavioral assays that had been previously used to assess depression-like symptoms in *Drosophila*. We first monitored the effect of chronic stress on courtship behavior (20). Courtship latency was quantified by measuring the time lag for the male to display the first wing extension. Compared with no treatment controls, chronically stressed flies showed significantly longer courtship latency (Fig. 1*D*), suggesting that these flies were less motivated to court a virgin female. We next investigated anhedonia-like behavior with the stop-for-sweet assay (13). Flies were allowed to stop and feed on a stripe of sweet-tasting glycerol while performing negative geotaxis. Chronically stressed flies made significantly fewer stops compared with controls, suggesting that they were lacking interest in enjoyment (Fig. 1*E*). Third, we tested forced swimming test (FST) (21), a classic behavioral assay for evaluation of antidepression activity. As shown in Fig. 1 *G* and *H*, after the 4-d CST, the latency to the first immobility period was significantly decreased while the duration of immobility was enhanced, indicating a tendency of despair-like behavior. Together with the finding that chronic stress induces learning and memory deficits, these data are consistent with previous reports that chronic stress could induce depression-like behaviors in *Drosophila* (13). To examine if our chronic stress procedure induces the formation of a long-lasting internal state, we tested olfactory learning at either 1 or 2 d ACS treatment. As shown in Fig. 1*I*, although the olfactory learning performance ACS is significantly lower than no treatment control, it is not significantly different from those tested in 1 d (1 d ACS) or 2 d (2 d ACS) later. We also tested FST at either 1 or 2 d ACS. Consistently, compared with no treatment control, the latency to immobility was significantly reduced when tested at each of the three time points (CST, 1 d ACS, or 2 d ACS) (Fig. 1*G*), while the duration of immobility was significantly increased (Fig. 1*H*). Therefore, the effect of chronic stress on these depression-like behaviors persisted for at least 2 d after treatment, suggesting that chronic stress induces a long-lasting depression-like state.

The DAergic System Modulates Susceptibility to CSLD. Since DAergic signaling is important for olfactory learning and responsive to vibration stimulus (22, 23), we reasoned that DAergic signaling might be important for the regulation of CSLD. To test this idea, we first took a pharmacological approach and blocked the rate-limiting enzyme for dopamine (DA) biosynthesis. We fed the flies with 3-iodotyrosine (3-IY), a tyrosine hydroxylase (TH)-specific inhibitor, in the last 2 d of the 4-d CST. Strikingly, down-regulating DA level during the process of CST significantly alleviated CSLD (Fig. 2*B*). Since DA is indispensable during training to form aversive olfactory memory, this counter-intuitive observation suggested that chronic stress–induced hyperdopaminergia could wreck the flies' capacity to form associative memory. We then fed the flies with L-DOPA (L-3,4-dihydroxyphenylalanine) plus carbidopa to increase brain DA level (24). Consistent with the above hypothesis, feeding L-DOPA/carbidopa in the last 2 d of CST further decreased the learning performance of chronically stressed flies (Fig. 2*C*).

Second, we examined whether the activity of Dop1R1, a D1-like DA receptor whose function is essential for olfactory learning, is important for CSLD. $Dop1R1^{dumb2}$ is a hypomorphic allele in which the expression of Dop1R1 in MBs is largely abolished (25). We used $Dop1R1^{dumb2/+}$ to down-regulate the expression level of Dop1R1 (25, 26). Untreated $Dop1R1^{dumb2/+}$ showed normal olfactory learning. However, when tested ACS treatment, $Dop1R1^{dumb2/+}$ animals exhibited significantly higher learning performance than wild-type controls (Fig. 2*D*), suggesting that reducing Dop1R1 level alleviates CSLD. These data also indicate that Dop1R1 hyperactivity might dampen olfactory learning. To verify this idea, we overexpressed Dop1R1 with Gal4 drivers driving *UAS-Dop1R1* in otherwise wild-type flies. Remarkably, overexpressing Dop1R1 with OK107, an MB-specific Gal4, driving *UAS-Dop1R1* resulted in a significant learning deficit (Fig. 2*E*). Adding an extra MB Gal4, R75F05, to drive even higher Dop1R1 expression further exacerbated the learning deficit (Fig. 2*E*), suggesting dosage sensitivity. Moreover, introducing an MB-Gal80 transgene into the above line effectively suppressed the learning deficit (Fig. 2*E*), indicating that Dop1R1 overexpression in MBs was necessary for inducing learning deficit. Importantly, sensorimotor responses to the odors or footshock used in the conditioning assay were not affected by Dop1R1 overexpression (*SI Appendix*, Fig. S6 *A–C*). Taken together, these data suggested that chronic stress–induced supernormal Dop1R1 activity in MBs precipitates susceptibility to CSLD.

The above experimental data support the idea that stress-induced hyperdopaminergic signaling during CST is important for the development of CSLD. However, both drug feeding and gene expression manipulation lacked fine temporal resolution. To directly test this hypothesis, we targeted genetically encoded tools to DANs for acute neural activity manipulation. To block neurotransmission from the majority of DANs, we used TH-Gal4 to drive $UAS\text{-}Shi^{TS1}$ that encodes a temperature-sensitive and dominant-negative mutant form of dynamin. Synaptic release from DANs could be conditionally inhibited by shifting to the restrictive temperature (27). As shown in Fig. 2 *F* and *G*, interrupting synaptic release from TH-labeled DANs only during the daily mechanical shock treatments was sufficient to prevent CSLD, while CSLD stayed significant if flies were shocked at permissive temperature (18 °C). These data suggest that stress-induced hyperactivity of DANs is

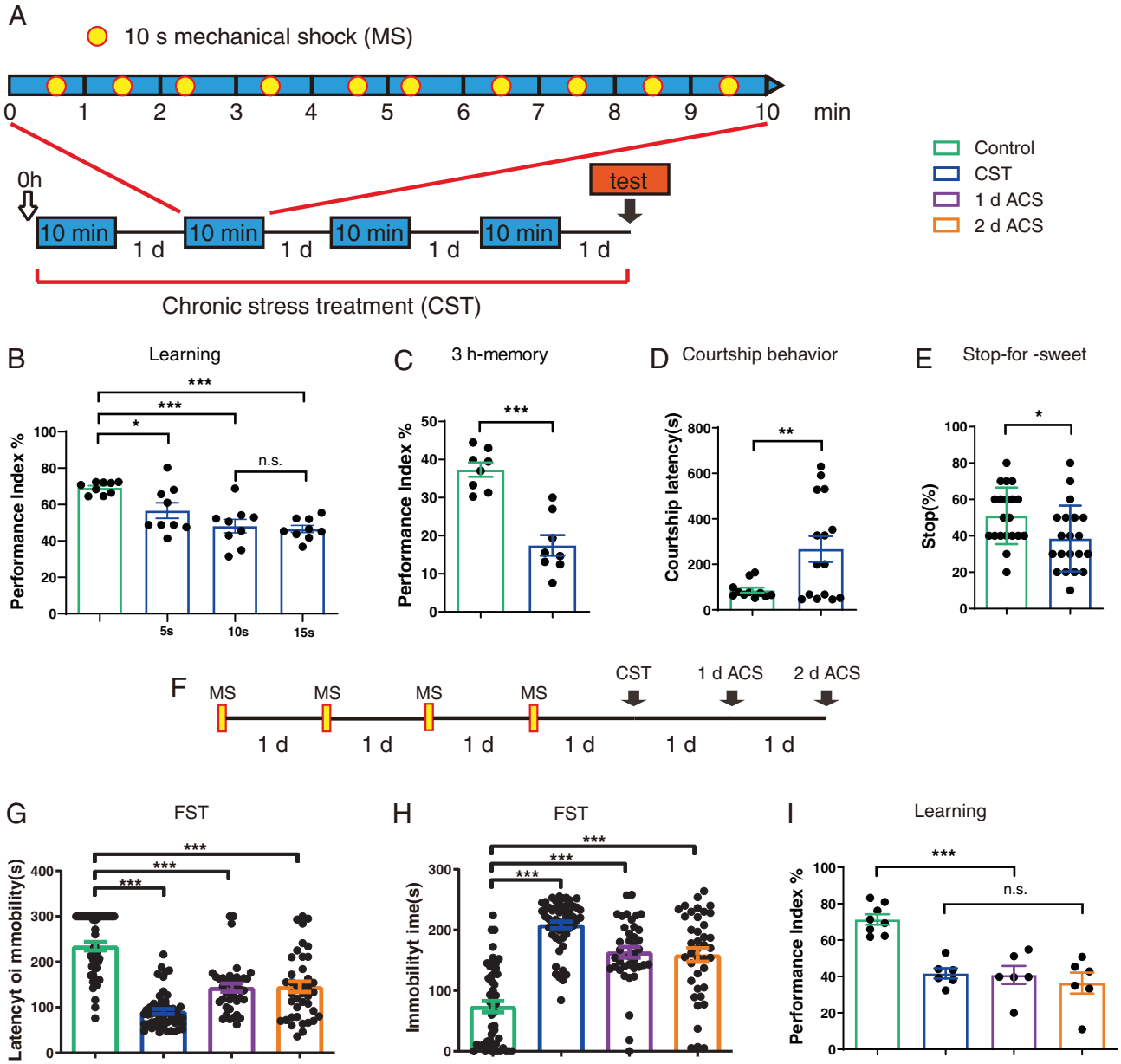

Fig. 1. Chronic stress induces olfactory learning and memory deficits as well as other depression-like behaviors in *Drosophila*. (*A*) Schematic of the standard CST procedure used in this study. During the 4-d CST, flies were cultured in small food vials and received 10 min of uncontrollable and unpredictable mechanical shock treatment each day. Behavioral assays were tested after treatment. (*B*) The effects of 4-d chronic stress with different vortex time on olfactory learning. Significant learning deficit was induced in each condition (one-way ANOVA, $F_{(3,\ 32)} = 11.75$, $P < 0.001$, $n = 9$; Tukey's test, for 5 s, $P < 0.05$; for 10 s, $P < 0.001$; for 15 s, $P < 0.001$). The learning deficit induced by 10-s vortex was not significantly different from that of 15-s vortex (Tukey's test, $P > 0.05$, $n = 9$). The 10-s vortex was chosen as the standard mechanical shock condition for the CSTs in the rest of this study unless otherwise noted. (*C*) Chronic stress induced significant 3-h memory deficit (*t* test, $P < 0.001$, $n = 8$). (*D*) The courtship latency was longer in chronically stressed males than controls (Welch corrected *t* test, $P < 0.01$, $n \geq 11$). (*E*) Chronically stressed flies tended to ignore the sweet substance in the stop-for-sweet assay (*t* test, $P < 0.05$, $n = 20$). (*F*) Schematic of the experimental design for *G*–*I*. In the FST, flies showed a long-lasting tendency of despair-like behavior, as indicated by shorter latency to immobility (*G*) and longer immobility time (*H*) ACS treatment, 1 d ACS, or 2 d ACS (for *G*, Kruskal–Wallis test, $H_{(3)} = 83.69$, $P < 0.001$; Dunn's test, $P < 0.001$; for *H*, Kruskal–Wallis test, $H_{(3)} = 76.86$, $P < 0.001$, $n \geq 39$ for all groups). (*I*) CSLD is long lasting, as the learning deficit could be detected not only after the 4-d CST but also at 1 d ACS or 2 d ACS (one-way ANOVA, $F_{(3,\ 22)} = 17.06$, $P < 0.001$, $n \geq 6$; Tukey's test, $P < 0.001$ for all groups). Moreover, the learning performances of these three time points are not significantly different from each other (Tukey's test, $P > 0.05$, $n = 6$ for all groups). Canton-S flies were used for courtship behavioral assays, while *w1118(isoCJ1)* flies were used for all the other experiments. Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); n.s., not significant. MS stands for mechanical shock; CST, chronic stress treatment; ACS, after chronic stress; FST, forced swimming test.

indispensable for chronic stress to erode olfactory learning. To examine if chronic activation of DANs could precipitate the development of CSLD, we drove the expression of the heat-activated ion channel TrpA1 (*UAS-TrpA1*) with TH-Gal4. In the course of CST, the mechanical shock was replaced with daily 1 h high-temperature exposure (30 °C) to activate the DANs. After 4 d of treatment, the learning performance of *TH-Gal4/UAS-TrpA1* flies was significantly decreased, whereas shock reactivity and odor acuity appeared normal (*SI Appendix*, Fig. S6 *D–F*), and *+/UAS-TrpA1* controls were not affected

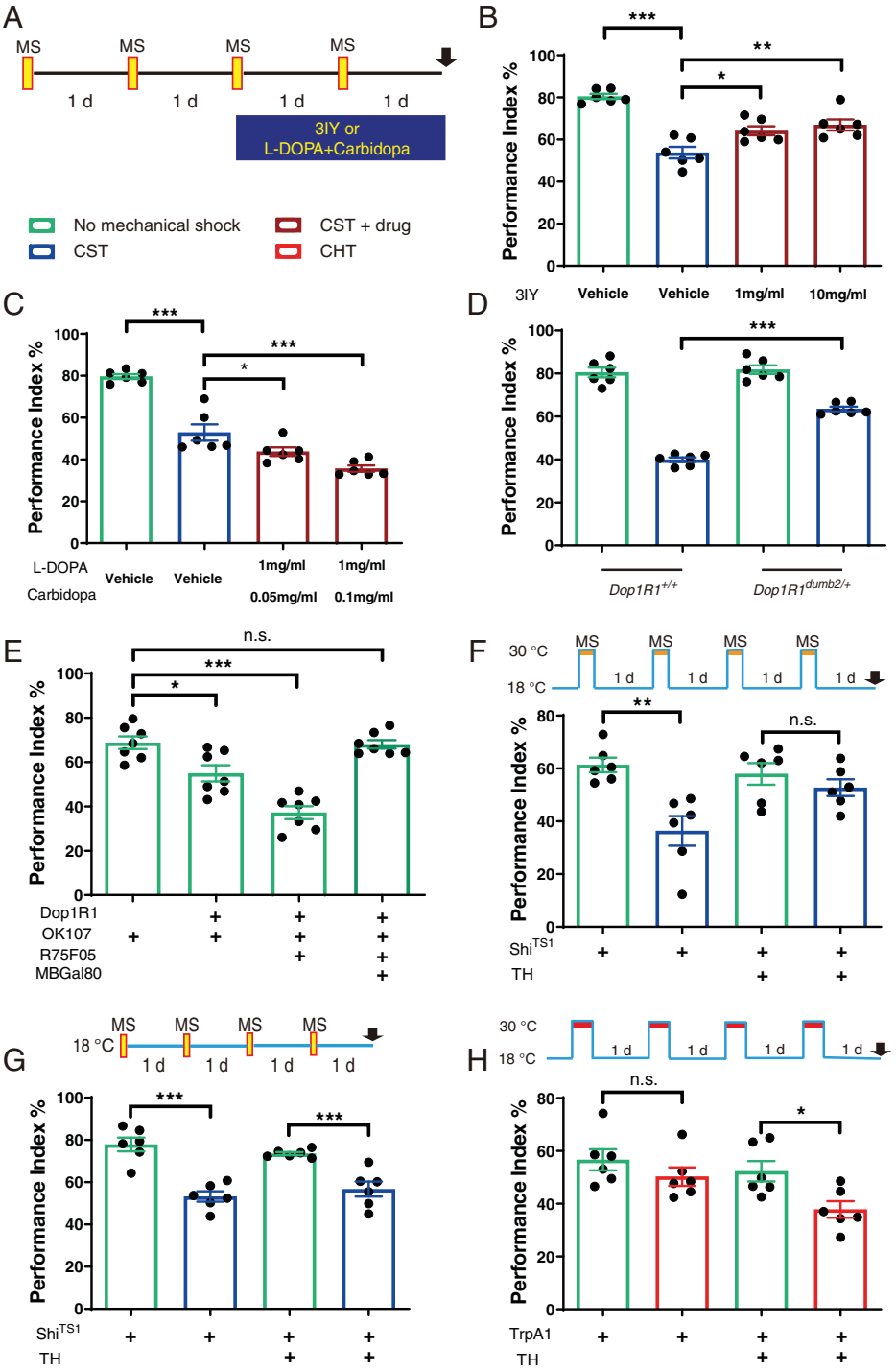

Fig. 2. Susceptibility to CSLD is modulated by dopaminergic system. (*A*) Schematic of the experimental design for *B* and *C*. (*B*) Feeding of DA synthesis inhibitor 3-IY in the last 2 d of the chronic stress procedure alleviated CSLD (one-way ANOVA, $F_{(3,\ 20)} = 23.34$, $P < 0.001$, $n = 6$; Dunnett's test, for 1 mg/mL, $P < 0.05$; for 10 mg/mL, $P < 0.01$). (*C*) Feeding of DA precursor L-DOPA plus carbidopa aggravated CSLD (one-way ANOVA, $F_{(3,\ 20)} = 63.78$, $P < 0.001$, $n = 6$; Dunnett's test, for 0.05 mg/mL carbidopa, $P < 0.05$; for 0.1 mg/mL carbidopa, $P < 0.001$). (*D*) *Dop1R1*$^{dumb2/+}$ flies showed a CSLD resistant phenotype (*t* test, $P < 0.001$, $n = 6$). (*E*) Overexpressing Dop1R1 with MB-specific Gal4 drivers in otherwise wild-type flies resulted in significant learning deficit (one-way ANOVA, $F_{(3,\ 24)} = 26.13$, $P < 0.001$, $n = 7$; Dunnett's test, for *OK107/UAS-Dop1R1*, $P < 0.05$; for *OK107, R75F05/UAS-Dop1R1*, $P < 0.001$), which can be rescued by adding an MB-Gal80 transgene (Dunnett's test, $P > 0.05$, $n = 7$). (*F*) Blocking synaptic release from TH-Gal4–labeled DANs during mechanical shock was sufficient to prevent CSLD (*t* test, $P > 0.05$, $n = 6$). (*G*) *TH/UAS-Shi*^{TS1} showed significant CSLD phenotype in permissive temperature (18 °C) (*t* test, $P < 0.001$, $n = 6$). (*H*) Replacing mechanical shock with thermogenetic activation of TH-labeled DANs by 1 h per day chronic heat treatment was sufficient to induce learning deficit (*t* test, $P < 0.05$, $n = 6$). Schematics that illustrate the experimental design are shown on the top of each panel. Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); n.s., not significant. MS stands for mechanical shock; CST, chronic stress treatment; CHT, chronic heat treatment; CSLD, chronic stress–induced learning deficit.

(Fig. 2*H*). Thus, excessive DANs activity during chronic stress is sufficient to induce learning deficit. Taken together, our experimental data on three levels, including DA, DA receptor, and DANs activity, are all in agreement with the notion that stress-induced excess DAergic activity promotes susceptibility to CSLD.

PPL1-γ1pedc Neurons Are the Key DANs That Precipitate Susceptibility to CSLD. The adult fly brain contains ∼280 DANs that project to diverse brain regions (28). To pinpoint the DAN subtypes that are involve in CSLD, we focused on two DAN clusters, PPL1 and PAM. Both of them project to MBs and are important for learning and memory. We used the Shi^{TS1} thermogenetic approach to acutely block DAN subsets during the mechanical shock treatments. R58E02-Gal4 marks the majority of PAM neurons (29, 30). Blocking PAM neurons with *R58E02-Gal4/UAS-*Shi^{TS1} showed no significant impact on CSLD (Fig. 3*B*), suggesting that the activity of PAM neurons is dispensable for CSLD. MB065B split Gal4 drives expression in a subset of PPL1 cluster neurons that project to the vertical lobes of MBs, including PPL1-γ2α′1, PPL1-α′2α2, PPL1-α3, and PPL1-α′3 (31, 32). Blocking these neurons with *MB065B/UAS-*Shi^{TS1} failed to alleviate CSLD as well (Fig. 3*C*). However, blocking neurotransmission with a PPL1-γ1pedc–specific split Gal4 MB320C driving *UAS-*Shi^{TS1} prevented CSLD (Fig. 3*D*). Furthermore, mechanical shock treatments performed at permissive temperature (18 °C) did not have a preventive effect on CSLD (Fig. 3*E*). These data suggest that PPL1-γ1pedc activity is indispensable for the development of CSLD. Besides strong expression in PPL1-γ1pedc neurons, MB320C split Gal4 also weakly marks PPL1-α′2α2 neurons (31, 33). To corroborate the indispensability of PPL1-γ1pedc for the development of CSLD, we drove *UAS-*Shi^{TS1} with VT50733, another PPL1-γ1pedc–specific driver that does not express in PPL1-α′2α2 neurons (*SI Appendix*, Fig. S7), and found that blocking PPL1-γ1pedc neurotransmission during mechanical shock with *VT50733-Gal4/UAS-*Shi^{TS1} also prevented CSLD (Fig. 3*F*). Importantly, chronic stress effectively induced learning deficit if these flies were shocked at permissive temperature (18 °C) (Fig. 3*G*). These data thus suggested that stress-induced PPL1-γ1pedc hyperactivity facilitates the development of CSLD. We verified this hypothesis by showing that chronic thermogenetic activation of PPL1-γ1pedc with *VT50733-Gal4/UAS-TrpA1* was sufficient to induce significant olfactory learning deficit in the absence of mechanical shock (Fig. 3*H*). Note that here we used a long protocol in which flies received daily 1-h thermogenetic activation in the first 2 d of the 4-d treatment followed by a 2-d constant thermogenetic activation. To confirm this finding, we used two additional PPL1-γ1pedc split Gal4 drivers, MB320C or MB438B (31), to chronically activate the neurons with the long thermogenetic activation protocol and showed that each was sufficient to induce a significant learning deficit in the absence of mechanical shock (Fig. 3*I*). Importantly, chronic thermogenetic activation of these neurons did not perturb sensorimotor responses to the odors or footshock (*SI Appendix*, Fig. S8 *G–O*). Based on these data, we concluded that PPL1-γ1pedc neurons are the key DANs whose activity mediates stress signals to precipitate susceptibility to CSLD.

MBON-γ1pedc>α/β Neurons Modulate Susceptibility to CSLD. PPL1-γ1pedc neurons project to the γ1pedc compartments of MBs, while MBON-γ1pedc>α/β neurons are the output neurons of γ1pedc compartments that furnish feedforward inhibition to multiple MBONs. Notably, PPL1-γ1pedc neural activity could depress MBON-γ1pedc>α/β activity (33, 34). We thus investigated whether MBON-γ1pedc>α/β neurons are important for regulating susceptibility to CSLD. Since stress-induced PPL1-γ1pedc activity precipitates susceptibility to CSLD, we hypothesized that stress-induced inhibition of MBON-γ1pedc>α/β activity should promote the development of CSLD. To test this hypothesis, we acutely activated MBON-γ1pedc>α/β with *R83A12-Gal4/UAS-TrpA1* (30, 34). Indeed, activating MBON-γ1pedc>α/β neurons only during mechanical shock treatments prevented CSLD (Fig. 4*B*), while CSLD appeared normal under permissive condition (18 °C) (Fig. 4*C*). On the other hand, chronic inhibition of MBON-γ1pedc>α/β synaptic release with *R83A12-Gal4/UAS-*Shi^{TS1} in the absence of mechanical shock was sufficient to induce a significant learning deficit (Fig. 4*D*), while shock reactivity and odor acuity were not diminished (*SI Appendix*, Fig. S9). These data suggested that stress-induced MBON-γ1pedc>α/β inhibition facilitates susceptibility to CSLD. Together with the above finding that PPL1-γ1pedc mediates stress signals to precipitate susceptibility to CSLD, our data indicated that the PPL1-γ1pedc–MBON-γ1pedc>α/β axis might be an important pathway for modulating susceptibility to CSLD.

Chronic Stress Induces Abnormal Neural Activity in the MB Network. Since normal MB function is required for olfactory learning, we speculated that chronic stress might induce abnormal neuronal activities in the MB network. To investigate this, we first took advantage of the CaLexA (calcium-dependent nuclear import of LexA) system that had been previously used to monitor accumulative neuronal activity in *Drosophila* (*SI Appendix*, Fig. S10 *A* and *B*) (35). CaLexA is composed of a genetically encoded GFP reporter whose expression is controlled by the intracellular calcium-dependent nuclear import of a chimeric transcription factor, the nuclear factor of activated T cells (NFAT) (35). We drove CaLexA and UAS-myr-tdTomato (to normalize the GFP signal) expression with MB subtype-specific Gal4s and focused on fluorescence in the lobe region (30, 36, 37). With VT30604, we found significantly reduced GFP/RFP signal in α′/β′ lobes of the chronically stressed flies compared with no treatment control (Fig. 5 *A* and *F*), indicating reduced accumulative neuronal activity in this brain region. However, when CaLexA was expressed via c739, the GFP/RFP signal in α/β lobes was not significantly different between chronic stress and control (*SI Appendix*, Fig. S10 *C* and *D*), suggesting that the neuronal activity of α/β does not change ACS. We used R14H06-Gal4 to drive CaLexA expression in γ-lobes and noted that the basal expression level between male and female flies was considerably different (30) (Fig. 5 *B* and *C*). We thus examined the γ-lobe GFP/RFP signal in male and female flies separately. Interestingly, significant enhancement was noted in the chronically stressed males compared with control (Fig. 5 *B* and *G*), yet no significant difference was detected in females (Fig. 5 *C* and *H*), suggesting sexual dimorphism of chronic stress response in this brain region.

In our attempts to investigate the neural activity of MB DANs and MBON-γ1pedc>α/β neurons, we noted that driving CaLexA with TH or MB112C, an MBON-γ1pedc>α/β–specific split Gal4, resulted in unreliably weak green fluorescent expression (31). To examine the impact of chronic stress on the activity of these neurons, we turned to ANF-GFP, a rat atrial natriuretic factor (ANF) and GFP fusion transgene that had previously been used to monitor neuropeptidergic vesicle trafficking and chronic neuronal activity (38–40). As neuronal excitation induces the release of the ANF-GFP containing vesicles and decreases GFP level in the terminal, the reduction of green fluorescence signal denotes increasing neuronal activity. To verify that the ANF-GFP signal reports the activity of neurons, we used TH to drive both ANF-GFP and TrpA1 in DANs and focused on γ1pedc, the MB compartment that PPL1-γ1pedc projects to. In line with previous reports, thermogenetic activation of DANs led to a significant reduction of ANF-GFP signal

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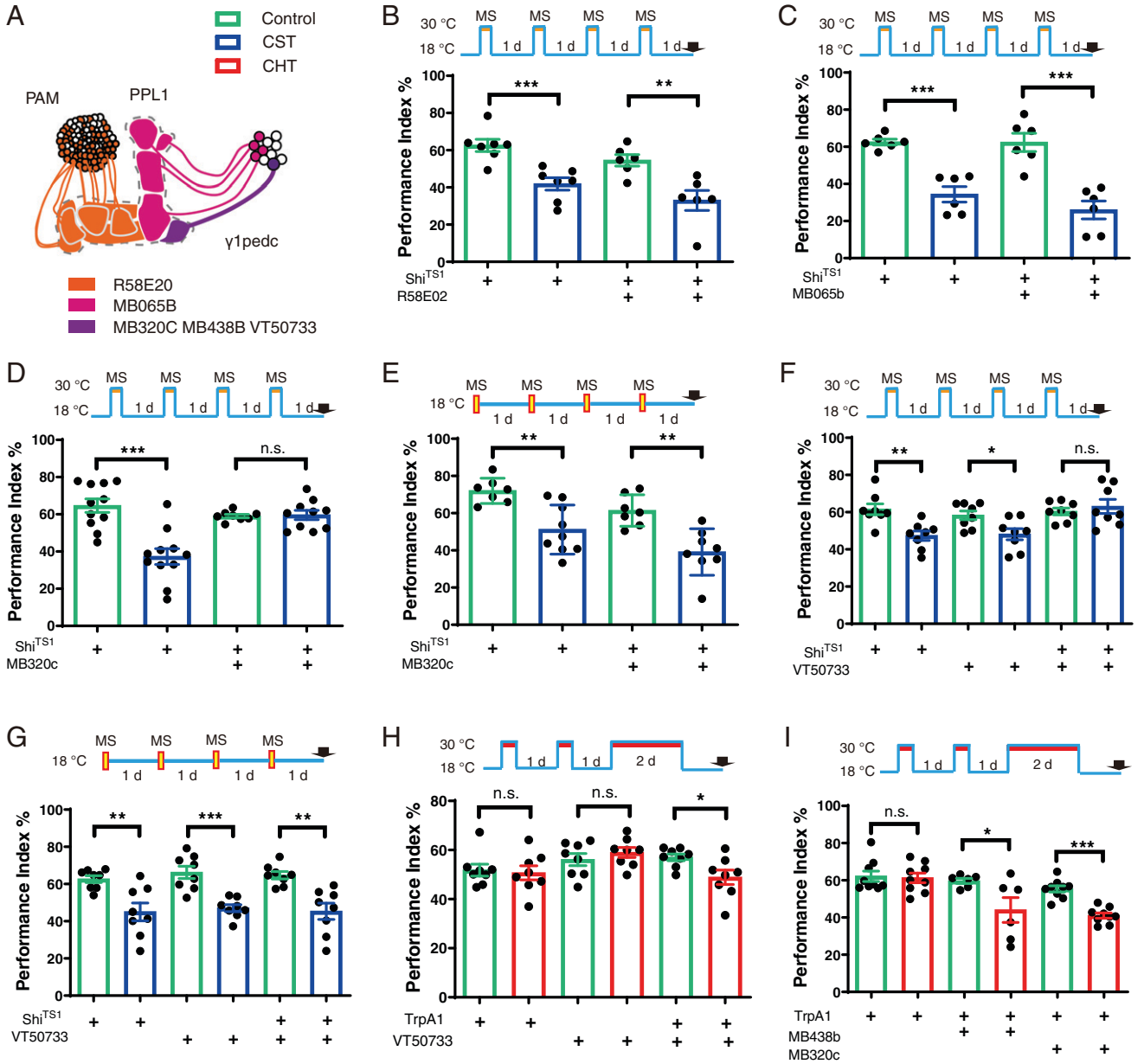

Fig. 3. The activity of PPL1-γ1pedc DANs provokes susceptibility to CSLD. (*A*) Schematic of expression patterns of Gal4 and Split-GAL4 drivers that each labels a subset of DANs that project to MB lobes. (*B*) Blocking synaptic release from PAM DANs with *R58E02-Gal4/UAS-Shi^{TS1}* could not prevent CSLD (*t* test, $P < 0.01$, $n = 6$). (*C*) Blocking synaptic release from PPL1-DANs that spare the γ1pedc subtype with *MB065B/UAS-Shi^{TS1}* could not prevent CSLD (*t* test, $P < 0.001$, $n = 6$). (*D*) In contrast, interrupting synaptic release from PPL1-γ1pedc DANs with *MB320C/UAS-Shi^{TS1}* only during mechanical shock treatments was sufficient to prevent CSLD (*t* test, $P > 0.05$, $n \geq 8$). (*E*) *MB320C/UAS-Shi^{TS1}* showed significant CSLD phenotype in permissive temperature (18 °C) (*t* test, $P < 0.01$, $n \geq 7$). (*F*) Interrupting synaptic release from PPL1-γ1pedc DANs with *VT50733/UAS-Shi^{TS1}* was also sufficient to prevent CSLD (*t* test, $P > 0.05$, $n \geq 8$). (*G*) *VT50733/UAS-Shi^{TS1}* showed significant CSLD phenotype in permissive temperature (18 °C) (*t* test, $P < 0.01$, $n = 8$). (*H*) Chronic activation of PPL1-γ1pedc with *VT50733/UAS-TrpA1* was sufficient to induce significant learning deficit in the absence of mechanical shock (*t* test, $P < 0.05$, $n = 8$). (*I*) Chronic activation of PPL1-γ1pedc with *MB438B/UAS-TrpA1* or *MB320C/UAS-TrpA1* was sufficient to induce significant learning deficit as well (*t* test, for *MB438B*, $P < 0.05$, $n = 6$; for *MB320C*, $P < 0.001$, $n = 9$). Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); n.s., not significant. MS stands for mechanical shock; CST, chronic stress treatment; CHT, chronic heat treatment; CSLD, chronic stress–induced learning deficit.

(38) (*SI Appendix*, Fig. S11 *A* and *C*). We then drove the expression of UAS-ANF-GFP and UAS-myr-tdTomato (to normalize the GFP signal) with TH and focused on the MB lobe region. TH-labeled DANs project to multiple MB compartments, including γ1pedc, γ2α′1, α2α′2, α′3, and α3 (28, 31). We examined each compartment separately. There was no significant difference in GFP/RFP signal between the chronic stress and control in each of the five compartments (*SI Appendix*, Fig. S11 *B* and *D*). These data suggest that the accumulative activity of DANs is not affected ACS. To probe MBON-γ1pedc>α/β neurons, we drove UAS-ANF-GFP and UAS-myr-tdTomato with MB112C (31). The axon of MBON-γ1pedc>α/β projects to α- and β-lobes. We found significant GFP/RFP signal reduction in the MBON-γ1pedc>α/β neuronal processes that project to the horizontal lobe region in the chronic stress group compared with no treatment control (Fig. 5 *D* and *I*), which may reflect

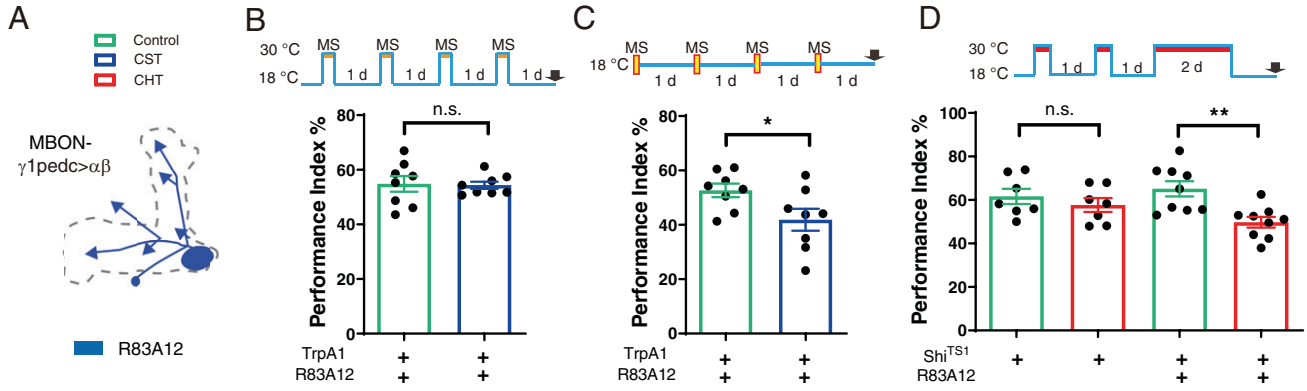

Fig. 4. MBON-γ1pedc>α/β neurons modulate susceptibility to CSLD. (*A*) Schematic of expression patterns of R83A12-Gal4 drivers that labels MBON-γ1pedc>α/β. (*B*) Thermogenetic activation of MBON-γ1pedc>α/β neurons only during the mechanical shock treatments prevented CSLD (*t* test, $P > 0.05$, $n = 8$). (*C*) The CSLD phenotype of *R83A12/UAS-TrpA1* under permissive condition (18 °C) was undiminished (*t* test, $P < 0.05$, $n = 8$). (*D*) Conversely, chronic interruption of MBON-γ1pedc>α/β synaptic transmission was sufficient to induce significant learning deficit in the absence of mechanical shock (*t* test, $P < 0.01$, $n = 9$). Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$); n.s., not significant. MS stands for mechanical shock; CST, chronic stress treatment; CHT, chronic heat treatment; CSLD, chronic stress–induced learning deficit.

an increased accumulative synaptic release from the MBON-γ1pedc>α/β to the MBONs in that region (34, 41).

Taken together, we identified abnormal neural activities in the MB network after flies had undergone CST. The abnormalities were remarkable especially in α′/β′ KCs and MBON-γ1pedc>α/β neurons, while aberration was also detected in the γ-KCs of male flies. Notably, these neurons all play important roles in olfactory learning, as blocking α′/β′ KCs synaptic release, activating γ KCs, or activating MBON-γ1pedc>α/β each could impair 3-min memory performance (34, 42–44). Our data thus demonstrate that chronic stress induces maladaptations in the MB network that are detrimental to olfactory learning.

DAergic Activity Is Indispensable for the Development of Chronic Stress–Induced Abnormal Neural Activity. Given that DAergic activity promotes susceptibility to CSLD and chronic stress induces abnormal neural activity in learning-related neurons, we predicted that DAergic activity should be important for the development of chronic stress–induced abnormal neural activity. To test this hypothesis, we blocked the biosynthesis of DA by feeding flies with 3-IY in the last 2 d of the chronic stress procedure and probed α′/β′ lobes with VT30604 driving CaLexA. As shown in Fig. 5 *E* and *J*, down-regulating DA level during the process of CST prevented the chronic stress–induced reduction of GFP/RFP signal in α′/β′ lobes, suggesting that DAergic activity is indispensable for chronic stress to induce abnormal neural activity in α′/β′ KCs. These data, together with all the above findings, are consistent with a model that stress-induced excess DAergic activity promotes susceptibility to CSLD by driving maladaptations in the MB network that lead to learning deficit.

Discussion

Depression-like symptoms in *Drosophila* can be induced by a variety of approaches, such as genetic manipulation, drug feeding, and stress treatments (12, 13, 45–49). Notably, a recent report has articulated that chronic stress could induce a depression-like state in *Drosophila* (13). In the report, a 3-d chronic vibration stress protocol induces depression-like behaviors and decreased serotonin activity. Moreover, these symptoms can be relieved by feeding antidepressant 5-hydroxy-L-tryptophan. Consistently, a later study reports that depression-like behaviors can also be induced by a 10-d chronic unpredictable mild stress paradigm and reverted by feeding fluoxetine (12). These findings suggest that chronic stress–induced *Drosophila* depression-like models could have great validity (50). In the present study, with a 4-d chronic stress protocol, we showed that CST could induce substantial learning and memory impairment, a core depression-like symptom not addressed in previous *Drosophila* chronic stress studies. Besides learning and memory impairment, other depression-like symptoms, such as lack of motivation, anhedonia, and prone to despair were also evidenced. Consistent with the idea that a depression-like state was induced, the learning deficit and prone to despair phenotypes appeared to be long lasting. Furthermore, we showed that stress-induced supranormal DAergic activity is a key etiologic factor for the development of CSLD, which nicely mirrors the important roles of the mammalian DAergic system in the etiology and maintaining of depression symptoms (51–54). Our findings thus provide additional evidence to corroborate the idea that *Drosophila* chronic stress paradigm could be a valid depression-like animal model.

Drosophila DAergic systems respond to various stress stimuli, including mechanical shock and electric shock (9, 22, 55, 56). To demonstrate DAergic activity is indispensable for the development of CSLD, we provided three lines of evidence: pharmacological manipulation of DA synthesis, genetic intervention of Dop1R1 function, and thermogenetic manipulation of DAN activity. All the manipulations were bidirectional, and the data consistently suggested that excess DAergic activity precipitates susceptibility to CSLD. Importantly, blocking DAN synaptic release only during mechanical shock prevents CSLD. Conversely, chronic DAN activation without mechanical shock is sufficient to induce a learning deficit. Since DAergic activity could be induced by vibration (22), these findings indicate that DANs might play an anxiogenic-like role and mediate chronic stress signals to provoke the development of learning deficit presumably by inducing allostatic maladaptation in learning-related neural circuits.

MB is the olfactory memory center in the fly central brain, and two clusters of DANs, PPL1 and PAM, send presynaptic projects to MB lobes. Among these DANs, we identified a pair of PPL1-γ1pedc neurons as the key DANs that are indispensable for evoking CSLD. Consistent with the presumptive function of relaying chronic stress signals to drive allostatic adaptation in the MB network, PPL1-γ1pedc neurons are known to respond to external noxious stimuli (such as electric shock, heat, and bitter taste) and mediate the aversive US for writing associative memory in MBs (28, 57–60). Furthermore,

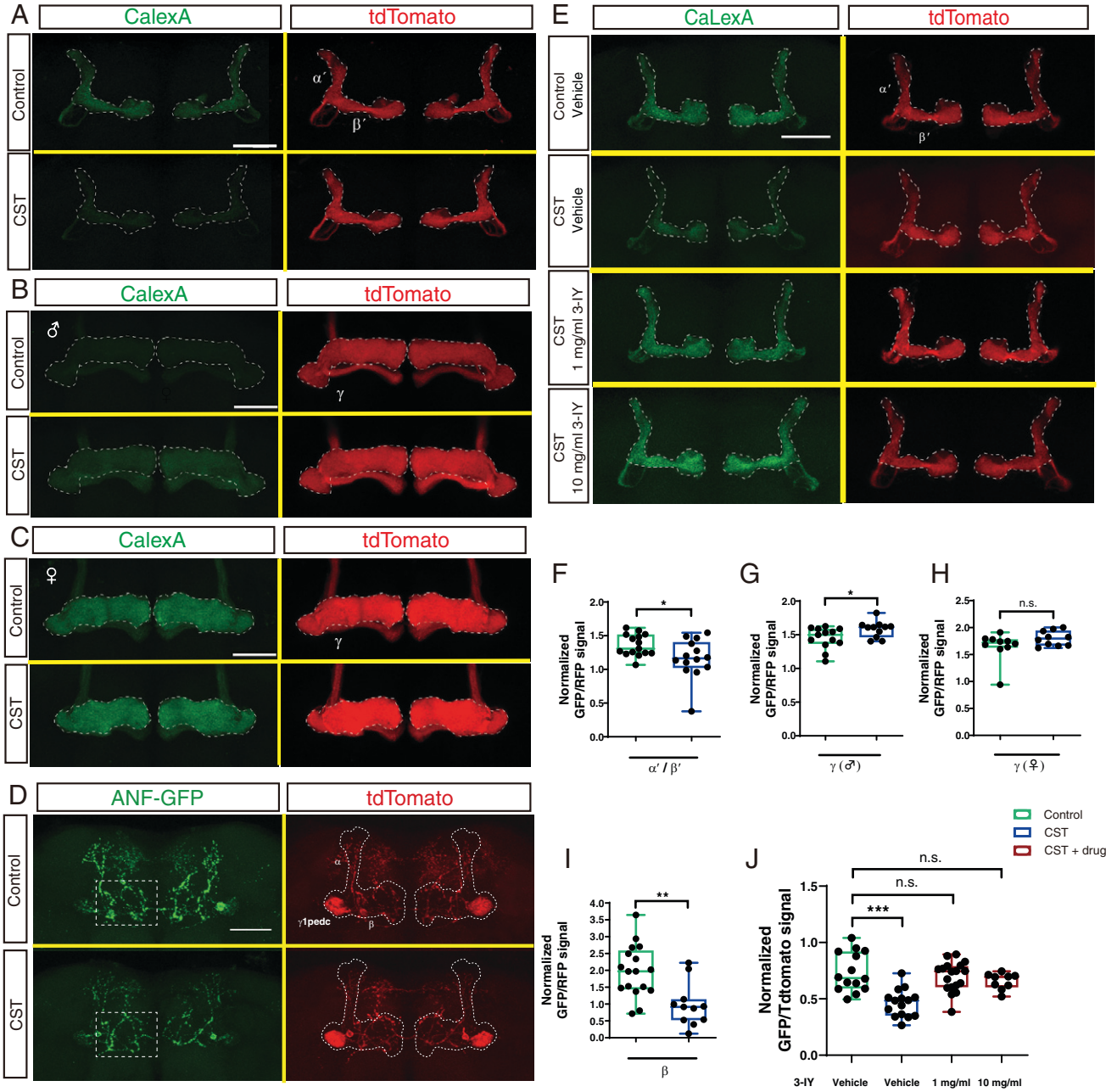

Fig. 5. Chronic stress induces abnormal neural activity in the MB network, which requires DAergic activity during CST. (*A*) Chronic stress induces the reduction of Ca^{2+} activity in α′/β′ lobes as measured with the CaLexA technique. Representative confocal images of CaLexA (*Left*) and tdTomato (*Right*) in the MB region of *VT30604-Gal4/CaLexA; UAS-myr-tdTomato* flies were shown. Dotted lines highlight MB α′/β′ lobes. (*B* and *C*) Chronic stress induces the increase of Ca^{2+} activity in the γ-lobes of male but not female flies as measured with CaLexA. Representative confocal images of CaLexA and tdTomato in the MB region of *R14H06-Gal4/CaLexA; UAS-myr-tdTomato* flies were shown in *B* for males and in *C* for females. Dotted lines highlight MB γ-lobes. (*D*) MBON-γ1pedc>α/β neurons are more active after the 4-d CST as indicated by diminished ANF-GFP signal in the β-lobe region. Representative confocal images of ANF-GFP (*Left*) and tdTomato (*Right*) in the MB area of *MB112C-Gal4/UAS-ANF-GFP; UAS-myr-tdTomato* flies were shown. Dotted lines highlight MB (*Right*) and β-lobe regions (*Left*). (*E*) Feeding DA synthesis inhibitor 3-IY in the last 2 d of the chronic stress procedure prevented chronic stress–induced Ca^{2+} activity reduction in α′/β′ lobes. Representative confocal images of CaLexA (*Left*) and tdTomato (*Right*) in the MB region of *VT30604-Gal4/CaLexA; UAS-myr-tdTomato* flies were shown. Dotted lines highlight MB α′/β′ lobes. (*F–J*) Quantitative analysis of normalized CaLexA fluorescence intensity in the region of α′/β′ lobes (*F*) (Welch corrected *t* test, $P < 0.05$, $n \geq 14$), γ-lobes (male) (*G*) (*t* test, $P < 0.05$, $n \geq 11$), and γ-lobes (female) (*H*) (Mann–Whitney *U* test, $P > 0.05$, $n = 10$). (*I*) Quantitative analysis of normalized ANF-GFP fluorescence intensity in the region of β-lobes (*t* test, $P < 0.01$, $n \geq 11$). (*J*) Quantitative analysis of the effect of 3-IY on the chronic stress–induced CaLexA signal reduction in the region of α′/β′ lobes (one-way ANOVA, $F(3, 52) = 12.52$, $P < 0.001$, $n \geq 9$; Dunnett's test, for CST, $P < 0.001$; for 1 mg/mL, $P > 0.05$; for 10 mg/mL, $P > 0.05$). In all confocal images, maximum projections of *Z*-stack sections with all the frames in the same parameter are shown. Scale bars in all images represent 50 μm unless otherwise noted. Box plots show the median (line inside the box), 25 and 75% quartiles (box), and minimum and maximum score (whiskers). The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); n.s., not significant. CST stands for chronic stress treatment.

PPL1-γ1pedc neurons display sustained rhythmic activity even in the absence of external stimulus, which has been implicated in the internal state (satiation) and memory processing after acquisition (8, 61). In the present paper, our imaging studies identified abnormal accumulative neural activities in the MB network of the chronically stressed flies, including reduced neural activity in α′/β′ KCs and enhanced neural activities in γ-KCs (male) and MBON-γ1pedc>α/β neurons (Fig. 5 *A*, *B*, *D*, *F*, *G*, and *I*). It is interesting to note that sleep deprivation also increases the spontaneous activity of γd KCs and decreases that of α′/β′ KCs, indicating that these neurons are vulnerable points on the MB network (62). As previously reported, either blocking α′/β′ KCs synaptic release, activating γ-KCs, or activating MBON-γ1pedc>α/β results in learning deficiency (34, 42–44). Thus, the superimposed effects of these chronic stress–induced maladaptations may well underlie CSLD. Importantly, similar to CSLD, the chronic stress–induced maladaptation in α′/β′ KCs also requires excessive DAergic activity during CST (Fig. 5 *E* and *J*). Thus, our findings support the model that PPL1-γ1pedc activity mediates chronic stress signals to drive allostatic maladaptations in the MB network that lead to a learning deficit.

Although further investigations are needed to fully understand how excess PPL1-γ1pedc activity induces maladaptations in the MB network, anatomical and functional connectivity suggest that PPL1-γ1pedc could directly activate or inhibit several neural cell types in the γ1pedc compartment, such as α/β KCs, γ KCs, and MBON-γ1pedc>α/β (31, 33, 34, 41, 63). These neurons could potentially relay PPL1-γ1pedc activity to other regions of the MB network so that PPL1-γ1pedc could broadly impact the MB network. GABAergic MBON-γ1pedc>α/β neurons, for instance, not only feedback to γ1pedc compartment but also send feedforward inhibition over MBONs of other compartments, which allows MBON-γ1pedc>α/β neurons to exert widespread impact on the MB network (41, 64, 65). As an example, the release of MBON-γ1pedc>α/β feedforward inhibition over MBON-γ5β′2a is implicated in aversive short term memory retrieval, and the MBON-γ5β′2a disinhibition, in turn, might activate PAM-γ5 DANs to form extinction memory (34, 66). Considering that stress-induced MBON-γ1pedc>α/β inhibition also facilitates susceptibility to CSLD (Fig. 4), the PPL1-γ1pedc–MBON-γ1pedc>α/β axis might play an important role in mediating the chronic stress signal to drive allostatic maladaptations in multiple MB compartments, which needs to be validated in future studies. Interestingly, accumulated stress-related memories (memories of stressful events) are believed to contribute to major depressive disorder (67). Since the PPL1-γ1pedc–MBON-γ1pedc>α/β axis is functionally important for memory formation (8, 57, 58, 64), modulations of the PPL1-γ1pedc–MBON-γ1pedc>α/β axis might have prevented CSLD by interrupting the vortex stress–related memory. Although this speculation still waits for a formal demonstration, it raises the possibility that the potential aversive vortex-odor associative memory formed during CST, if any, might interfere with later olfactory conditioning and contribute to CSLD. Testing whether DA signaling is also important for developing other chronic stress–induced depressive-like behavior would help to resolve this issue. We thus examined the forced swimming phenotype after overexpressing Dop1R1 with MB-specific GAL-4 drivers. As shown in *SI Appendix*, Fig. S13, Dop1R1 hyperactivity in MBs did not affect latency to immobility and total immobility time in the FST, suggesting independent mechanisms are involved.

The gross DA level in the fly head is remarkably downregulated following chronic unpredictable mild stress (12). Similarly, our 4-d CST also leads to diminished DA concentration in the fly head (*SI Appendix*, Fig. S12), indicating that DAergic activity might be attenuated ACS. Consistent with this idea, imaging studies indicate that the inhibitory feedback to PPL1-γ1pedc from GABAergic MBON-γ1pedc>α/β neurons might be enhanced ACS treatment (64) (Fig. 5 *D* and *I*). Therefore, it is surprising that no change of accumulative spontaneous activity was detected in the PPL1 neurons that project to MB. Interestingly, in rodents, the effects of chronic stress on the ventral tegmental area (VTA) DAN activity depend on chronic stress protocols, even though all the protocols are capable to induce depression-like symptoms (68). Generally, CSTs using relatively mild stressors, such as cold or mild food shock, tend to induce long-lasting attenuated spontaneous VTA DAN activity, while those using more severe stressors, such as social defeat, are prone to induce enhanced spontaneous VTA DAN activity (51, 69, 70). These indicate that complex mechanisms underlie depression-associated maladaptations in DANs. We, therefore, speculate that ANF-GFP might not be sensitive enough to detect the chronic stress–induced change in DANs, or maladaptive response of the DAergic system might involve mechanisms other than DAN spontaneous activity in *Drosophila*.

Drosophila genome encodes 4 DA receptors, Dop1R1, Dop1R2, Dop2R, and DopEcR. Dop1R1 is highly expressed in MB and indispensable for aversive olfactory learning and memory (25, 71). A previous study has suggested an "inverted-U" dosage response of Dop1R1 level on long term memory (72). In the current study, we show that a similar Dop1R1 "inverted-U" dosage relationship also applies to learning, as Dop1R1 overexpression in MB KCs results in olfactory learning impairment (Fig. 2*E*). Importantly, down-regulating Dop1R1 expression with $Dop1R1^{dumb2/+}$ alleviates CSLD (Fig. 2*D*), suggesting that excess Dop1R1 signaling in KCs is a key determinant of susceptibility to CSLD. A similar inverted U–shaped dose–response relationship between DA receptor D1 (D1R) and working memory is well known in the mammalian prefrontal cortex (73). And stress-induced supranormal D1R activity could markedly impair working memory (74, 75). Furthermore, in the mammalian amygdala, DA also mediates anxiety via D1R and D2R (76). These similarities suggest that the anxiogenic effects of D1R activity might have evolutionarily conserved mechanisms. Whether Dop1R2, Dop2R, and DopEcR are involved in CSLD remains an open question.

Collectively, in this study, we establish a *Drosophila* model for studying the impact of chronic stress on learning and memory. Our investigation of the etiology of CSLD has demonstrated that the DAergic system plays essential roles in modulating susceptibility. Especially, we identified a single pair of DANs, PPL1-γ1pedc, that mediate stress signals to induce allostatic overload of the MB network that results in abnormal neural activities and learning deficits. These suggest that with abundant genetic tools and complete connectome, the *Drosophila* model can provide a unique opportunity to study conserved signaling pathway mechanisms underlying chronic stress–induced cognitive impairments in a learning and memory center whose anatomy is well-annotated.

Materials and Methods

Fly Stocks. Flies were cultured in cornmeal fly food at 23 °C. The following fly stocks were used in this study: *w1118(isoCJ1)*, Canton-S, NP2758, Elav, TH, OK107, c739, *UAS-myr::GFP*, *UAS-Shibire^{TS1}*, and *UAS-TrpA1*. The following FlyLight Split-GAL4 lines were shared by Yi Zhong, Tsinghua University, Beijing: MB065B, MB320C, MB438B, MB131B, and MB112C (31). *Dop1R1^{dumb2}*, *UAS-Dop1R1*, and MBGal80 were gifts from Josh Dubnau, Stony Brook University, New York. CaLexA (*LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP16-NFAT, lexAop-rCD2-GFP*) was from Zhefeng Gong, Zhejiang University, Hangzhou. *UAS-ANF-GFP*, *UAS-myr-tdTomato,* R58E02, R75F05, R83A12, and R14H06 were ordered from Bloomington *Drosophila* Stock Center (BDSC). VT30604, VT49246, and VT50733 were ordered from Vienna *Drosophila* Resource Center. *w1118(isoCJ1)*, a white isogenic line derived from Canton-S, was used as the wild-type control unless otherwise noted. *Dop1R1^{dumb2}*, *UAS-Dop1R1*, MBGal80, *UAS-Shibire^{TS1}*, *UAS-TrpA1*, Elav, TH, OK107, and C739 strains had been equilibrated to *w1118(isoCJ1)* for at least five generations before being utilized.

CST. Groups of about 100 flies were collected and cultured in small food vials (restricted in a space of 25 mm in diameter and about 55 mm in height). During the CST, flies received a 10-min mechanical shock every day. Before the mechanical shock, flies were transferred into empty 10-mL centrifuge tubes (16 mm in diameter). The tubes were put on the perforated foam cushion installed on a vortex shaker (IKA GENIUS 3). Vortexes of 500 rpm were applied for 10 s in each minute of the 10-min treatment. To avoid habituation, vortexes were started randomly within the minute. After the 10-min vortex, flies were transferred back to the food vials. The same treatments were repeated for 4 consecutive days. Behavioral assays were tested at the end of the 4-d treatment (1 d after the last vortex).

Behavior Assays. All behavioral experiments were carried out in an environmental chamber with 70% humidity. The temperature was set at 25 °C except for Shibire^{TS1} and TrpA1 experiments.

For aversive olfactory learning and memory, flies were trained to associate odors with electric shock in a T-maze apparatus with a Pavlovian conditioning paradigm as previously described (15, 16, 71). Each individual *n* consisted of ~200 flies, with half of the flies trained to one odor and half to the other odor. For learning, flies were trained with a single training session and tested immediately after training. For 3-h memory, flies were transferred into food vials after training and kept in dark for 3 h before testing. For odor acuity, odor avoidance to 3-octanol (OCT, Sigma-Aldrich, product no. 218405) or 4-methylcyclohexanol (MCH; Sigma-Aldrich, product no. 218405) was quantified as previously described (15, 16, 77). For shock reactivity, shock avoidance to 60 V was quantified as previously described (15, 16, 77).

Male courtship assay was adapted from a previous report (20). Briefly, 6- to 8-d-old stressed or control virgin flies were cold anesthetized and individually loaded into two-layer round chambers (diameter: 1cm; height: 2.5 mm per layer, a gift from Yufeng Pan, Southeast University, Nanjing). To let flies adapt to the chamber environment, males were separated from target females by a transparent plastic barrier for 1 h before the courtship test. Courtship tests were videoed with a camcorder (Canon HF R806). The time lag to the first courtship display by the male after pairing with the female was recorded.

FST was adapted from a previous report (21). Briefly, a single fly was gently aspirated into a chamber (35 × 10 mm) filled with 2.5 mm height of 0.08% sodium dodecyl sulfate solution (Sangon Biotech, product no. A600485). Each fly was videotaped for 5 min (Canon HF R806). Latency to the first period of immobility and duration of immobility were recorded for final analyses.

Stop-for-sweet was tested according to previous description (13). In brief, qualitative filter papers (medium speed) were cut into rectangles of 55 × 20 mm. Along the midline of the paper, 5-mm-wide traces of glycerol (Sinopharm, product no. 10010618) were painted. The papers were held upright in a chamber with an angle of 110° to 120°. Stressed or no-stressed control flies, with their wings cut before test, were allowed to climb up the filter papers and stop to eat when they walked by the glycerol. Each fly was tested 10 times, and the number of stops was scored.

Confocal Microscopy. Brain samples were prepared as previously described (71). Adult fly brains were dissected in cold phosphate buffer saline (PBS) and then fixed in 4% paraformaldehyde for 20 min. Fixed brains were transferred into phosphate buffer saline with 0.5% Triton X-100 (PBST) and vacuum twice in a vacuum desiccator, 10 min each. Brains were mounted in VECTASHIELD mounting medium (Vector Laboratories, Inc.). The confocal images were acquired with an Olympus FV1200 or a Nikon TI-E+A1 SI confocal microscope. The image data were processed with ImageJ or Fiji ImageJ and later manipulated as figures in Adobe Photoshop and Illustrator (Adobe Systems Incorporated). To measure fluorescent intensities, we manually selected the region of interest (ROI) and measured the total intensity. To correct for background, background fluorescent intensity from the adjacent area of the ROI was measured and subtracted. To compare CaLexA or ANF-GFP signals between individual flies, the GFP signals were normalized to the tdTomato signals from the same ROIs: $F = (F_{gfp} - F_{background\text{-}gfp})/(F_{tdtomato}-F_{background\text{-}tdtomato})$.

Pharmacology. 3-IY (I8250, Sigma), L-DOPA (D9628, Sigma), or S-(−)-Carbidopa (C1335, Sigma) were dissolved in 5% sucrose plus 2% yeast solution. For drug feeding, adult flies were transferred to food vials containing a tissue (4 × 4 cm) soaked with 2 mL of the sucrose solution. Control flies were fed with vehicle (5% sucrose plus 2% yeast solution) for the same amount of time.

TUNEL Staining. Dissected fly brains were treated with 20 μg/L proteinase K (Sangon Biotech, Shanghai) in PBS buffer for 25 min. After washing three times with PBST, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining was done by following the instruction from the manufacturer (In Situ Cell Death Detection Kit, Fluorescein, Roche).

Smurf Assay. Smurf assay was adapted from a previous report (78). Flies were maintained on standard corn meal medium containing 2.5% brilliant blue dye (Shanghai Dyestyffs Research Institute Co., Ltd) for 1 d. Smurf phenotype was determined if the blue color was observed outside of the digestive tract.

Enzyme-Linked Immunosorbent Assay. For each independent experiment, the heads of 200 flies were collected and homogenized in PBS (pH 7.2). The samples were then centrifuged at 10,000 *g* for 20 min at 4 °C, and supernatants were collected. Enzyme-linked immunosorbent assays (ELISAs) were done following the instruction from the manufacturer (Cloud-Clone Corp., product no. CEA851Ge). A multimode plate reader (PerkinElmer EnSpire) was used to detect the optical intensities.

Statistics. Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software). According to the central limit theorem, the performance indices (PIs) of the T-maze assays (the average of two half PIs) should be normally distributed (79, 80). For the rest of the data in this study, the normality of data were determined with the D'Agostino and Pearson test. For data sets that are normally distributed, Student's *t* test or Welch corrected *t* test was used for comparisons between two groups, and one-way ANOVA followed by Tukey's test or Dunnett's test was used for comparisons of multiple groups. For data sets that are not normally distributed, Mann–Whitney *U* test was used for comparisons between two groups, and Kruskal–Wallis test followed by Dunn's test was used for comparisons of multiple groups. The threshold for statistical significance was set at $P < 0.05$. In all bar graphs, data are presented as means ± SEM. Box plots show the median (line inside the box), 25 and 75% quartiles (box), minimum and maximum score (whiskers).

Data Availability. All study data are included in the article and/or supporting information.

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